CELECOXIB ENHANCES THE RADIOTHERAPY OF SECONDARY BONE TUMORS OF A HUMAN NON-SMALL CELL LUNG CANCER VIA ANTI-ANGIOGENESIS IN VIVO

1 Frank Michael Klenke, 3 Amir Abdollahi, 3 Heike Zieher, 4 Martha-Maria Gebhard, 5 Volker Ewerbeck, 3 Peter Huber, 2 Axel Sckell

1 Department of Orthopedic Surgery, Inselspital, University of Berne, CH-3010 Berne, Switzerland

2 Department of Trauma and Reconstructive Surgery, Charité - Campus Benjamin Franklin, Hindenburgdamm 30, D-12200 Berlin, Germany

3 German Cancer Research Center, Department of Radiation Oncology, INF 280, D-69120 Heidelberg, Germany

4 Department of Experimental Surgery, University of Heidelberg, INF 365, D-69120 Heidelberg, Germany

5 Department of Orthopedic Surgery, University of Heidelberg, Schlierbacher Landstrasse 120a, D-69118 Heidelberg, Germany

Submitted for publication
ABSTRACT

Together with the surgical stabilization or reconstruction of skeletal regions affected by metastatic cancer, radiotherapy continues to remain the major treatment modality for bone metastases. However, a long-term disease control is usually not achieved. Cyclooxygenase-2 (cox-2) inhibitors mediate a systemic anti-tumor activity via anti-angiogenesis and seem to enhance the response of primary tumors to radiation. Radio sensitizing effects of cyclooxygenase-2 inhibition have not been reported for bone metastases. Therefore, the aim of this study was the investigation of the radio sensitizing effects of the selective Cox-2 inhibitor celecoxib in secondary bone tumors of a non-small cell lung carcinoma in vivo. A549 lung carcinomas were implanted into a cranial window preparation in male Scid mice (n=24). Animals were treated with either celecoxib or radiation alone or a combination of celecoxib and radiation, respectively. Untreated animal served as control. The impact of radiation and cox-2 inhibition on angiogenesis, microcirculation and tumor growth was analyzed over 28 days by means of intravital microscopy and histological methods. Monotherapies with radiation as well as celecoxib had significant antitumor effects compared to untreated controls. Both therapies reduced tumor growth and vascularization to a similar extent. The simultaneous administration of celecoxib and radiation further enhanced the antitumor and anti-angiogenic effects of single-beam radiation. With the combined treatment approach tumor vascularization and tumor size were decreased by 57% and 51%, respectively as compared to monotherapy with radiation. The combined application of radiation therapy and cox-2 inhibition showed synergistic effects concerning the inhibition of tumor growth and tumor angiogenesis. Therefore, the combination of radiation with cox-2 inhibitor therapy represents a promising approach to improve the therapeutic efficacy of radiation of bone metastases.

BACKGROUND AND PURPOSE

The skeleton is one of the most common organs to be affected by metastatic cancer. Primary tumors arising from the breast, prostate, thyroid, lung, and kidney show a special predilection to spread to bone 1. Bone metastases have substantial negative effects on the patient's quality of life. The decline in quality of life is often due to pathological fractures and their implications such as pain.
and neurological deficits and is not necessarily caused by the primary tumor itself.

The standard treatment of bone metastases consists in radiation of the affected skeletal region to prevent disease progression and fracture. As the response rate of tumors to radiation seems to be dependent on the delivered dose, strategies increasing the effective dose of radiation may be crucial to ameliorate the therapeutic efficacy of radiotherapy \(^2-7\). Recent strategies to optimize the efficacy of radiation are focused on molecular targets enhancing the radiation sensitivity of malignant tumors \(^8-18\). In this respect, the prostaglandin signaling pathway seems to be of particular importance as it has been shown that the modulation of prostaglandin synthesis can ameliorate the response of tumors to radiation \(^13,14,19,20\).

Cyclooxygenase (cox) with its two isoforms cox-1 and cox-2 is the rate-limiting enzyme for the synthesis of prostaglandins from free arachidonic acids. Cox-1 is constitutively expressed in most normal tissues and is responsible for the production of prostaglandins that mediate regular physiological functions. The inducible isoform cox-2 is usually undetectable in normal tissue and is frequently overexpressed in malignant and inflamed tissues \(^21,22\). Elevated levels of cox-2 in tumor cells are associated with resistance to apoptosis \(^23,24\), tumor angiogenesis \(^25\), and tumor cell invasiveness \(^26-28\). It has been shown that the inhibition of cox-2 mediates antitumor activities in various human malignant tissues including prostate colorectal, breast, and non-small cell lung cancer. \(^29-35\). In a previous study it was shown that the selective cyclooxygenase-2 inhibitor celecoxib significantly reduced growth of secondary bone tumors of a non-small cell lung carcinoma. The anti-tumor effect was mediated by antiangiogenic and pro-apoptotic mechanisms in bone metastases \(^36\).

Although cox-2 inhibitors were shown to inhibit tumor growth if administered as monotherapeutic agents, several authors provided evidence that the drugs are considerably more effective if combined with a second treatment option. In this regard, selective cox-2 inhibitors were recently reported to enhance the response of primary tumors to radiation \textit{in vitro} and \textit{in vivo} \(^13,14,19,20,37-39\). The therapeutic efficacy of a combination of cox-2 inhibition and radiation on secondary bone tumors has not been described so far. We hypothesized that the selective cox-2 inhibitor celecoxib may enhance the radioresponse of secondary bone tumors of a non-small cell lung carcinoma \textit{in vivo}. The effects of the combined application
of celecoxib and radiation were investigated by applying an animal model of bone metastases and intravital microscopy to continuously monitor angiogenesis, vascularization, and growth of secondary bone tumors

**MATERIAL AND METHODS**

*Animal model and cell lines*

Experiments were performed on 24 adult male severe combined immuno deficient mice (SCID, C.B-17/IcrCrl-scid-BR, Charles River Laboratories Inc., Sulzfeld, Germany, 7 to 8 weeks old, 20 to 25 g body weight), following institutional guidelines approved by the local animal review board. All surgical procedures were performed in strictly aseptic conditions within a laminar flow unit (Merck Eurolab, Bruchsal, Germany) under deep anesthesia by an intraperitoneal injection of a mixture of ketamine (Ketanest®, 65 mg/kg body weight, Pfizer, Karlsruhe, Germany), xylazine (Rompun®, 13 mg/kg body weight, Bayer, Leverkusen, Germany) and acepromazine (Sedastress®, 2 mg/kg body weight, Medistar, Holzwickede, Germany).

The human lung carcinoma cell line A 549 was obtained from the German Cancer Research Institute (Heidelberg, Germany). Tumor cells [1 x 10⁷/ml] were injected subcutaneously into the left flank of a donor mouse each and grown to a volume of 0.5 to 1.0 cm³. After sacrificing the donor mouse, the tumor was excised, cut into small pieces (volume 0.5-1.0 mm³) in Dulbecco’s Modified Eagle’s Medium (DMEM) at 4°C and implanted into the recipient mouse in the following manner:

Surgical preparation of the cranial window was performed as described in detail elsewhere. Briefly described, the scalp of the mouse was shaved and surgically excised in an oval area to expose the frontal and parietal bone. The periosteum was removed and an oval cavity of approximately 2 mm by 1 mm by 0.5 mm was milled into the calvaria eliminating parts of the external tabula of the calvaria including the spongy bone underneath. Then one small piece (approx. 0.5-1.0 mm³) of the human non-small cell lung carcinoma A549 was implanted into the cavity. To prevent dehydration or mechanical damage to the tumors, the preparation was sealed with a glass cover slip and bone cement made of a mixture of ethyl cyanoacrylate glue (Pattex® Blitz Kleber, Henkel, Germany) and GC Ostron®-Powder (methyl methacrylate polymer, GC Europe,
Belgium). The animals were held individually in special filter cages to maintain aseptic conditions and to prevent mutual damage to the cranial window. The animals were provided with sterile standard pellet food and water ad libitum.

**Radiation therapy and cox-2 inhibitor treatment**

Radiotherapy was delivered on day 15 after tumor implantation with a single shot dose of 7 Gray (Gy) \( \gamma \) -radiation to the cranium using a Co-60 source (Siemens, Gammatron, Erlangen, Germany). The selective Cox-2 inhibitor celecoxib was a generous gift of Pharmacia Inc. (St. Louis, MO, USA). Celecoxib was dissolved in a carboxymethylcellulose (CMC)-based vehicle at 5 mg celecoxib/ml vehicle. Animals each were treated once daily by s.c. injection of 30 mg/kg body weight celecoxib (Celecoxib, n=6) or the equivalent amount of the (CMC)-based vehicle alone (Control, n=6). Animals that received radiation on day 15 were either treated with the vehicle (Radiation, n=6) or with celecoxib (Celecoxib + Radiation, n=6) under the conditions described above. Treatments started on day 8 after tumor implantation and were continued until termination of experiments on day 28 after tumor implantation. The injection of celecoxib or CMC-vehicle alone and the single shot irradiation to the cranium were well tolerated, no difference in animal behavior or loss of weight was observed.

**Intravital microscopy**

For intravital microscopy, mice were anesthetized and positioned on a custom made stereotactic device. Within the first week after tumor implantation, mice were observed daily under epi-illumination with a stereotactic microscope (Leica MZ7s, Leica, Germany) employing a 5 to 40-fold magnification. At 24 hour intervals, the first appearance of (i) hemorrhage, (ii) the first appearance of newly formed blood vessels entering the implanted tumor tissue, and (iii) the onset of perfusion in these newly formed vessels were determined. Intravital fluorescence video microscopy was performed using an epi-illumination fluorescence microscope unit (Leica, Germany) equipped with a 4x (EF 4/0.12, Leitz, Wetzlar, Germany) and 40x (Zeiss Achromplan 40x/0.75 w, Carl Zeiss, Germany) objective on days 7, 14, 21 and 28 after tumor implantation. For off line analysis, regions of interest were recorded on videotapes using a S-VHS videocassette recorder (AG-7350, Panasonic, Japan) at a rate of 50 frames/s and a digital camera (Kappa CF 8/1, Kappa Opto-electronics, Germany). Using an
adequate fluorescence filter set for green light (bandpass 515-560 nm), the intravenous injection of fluorescein isothiocyanate (FITC)-labeled dextran (Sigma, St. Louis, MO, FITC-Dextran, FD 2000S, molecular weight 2.000.000; 0.1 ml of a 5% solution in 0.9% NaCl as a plasma marker) enabled the observation of the tumor microcirculation.

**Off-Line analysis of tumor growth and microhemodynamics**

Tumor growth was determined *off-line* by measuring its two-dimensional surface area in mm² from standardized digital photographs of the cranial window preparation at 10-fold magnification on days 7, 14, 21, and 28 after implantation using a computer based analysis program (AnalySIS® V3.0, Soft Imaging System, Münster, Germany). The functional microvessel density (*FVD*) was determined as the length of all perfused microvessels within a tumor in relation to the two-dimensional surface area of the tumor in mm/mm² indicated by the fluorescence of FITC labeled dextran in all perfused vessels. Recordings on videotape for *off line* analysis of the FVD were made for 15 s each. The *off line* analysis was performed using a computer based image analysis program (CapImage®, Engineering Office Dr. Zeintl, Heidelberg, Germany).

**Histopathologic assessment**

The mice were sacrificed on day 28 after tumor implantation and the tumors were immediately excised along with the surrounding tissue of the calvaria and the brain for further histopathologic investigation. Tissue samples were fixed for 24-48 hours by immersion in 4% formalin solution. After decalcification of the bone in ethylene-diaminetetraacetic acid for 2 weeks, samples were embedded in paraffin and sliced into three-µm serial sections for Hematoxylin-Eosin staining.

**Statistics**

All numerical data are presented as median with 25% and 75% quartiles. Using the software program SigmaStat® for Windows (Version 2.03, SPSS, Chicago, IL), data were analyzed statistically with ANOVA on ranks. The Student-Newman-Keuls Method was applied for multiple comparison procedures. Differences were considered significant at p<0.05.
RESULTS

Tumor growth

As shown in figure 1, Tumor growth was identical in all groups before the treatment with celecoxib or the vehicle control was initiated: control: 0.84 mm$^2$ [0.78/1.15], celecoxib: 0.81 mm$^2$ (0.68/1.05), radiation: 0.82 mm$^2$ (0.77/0.91), celecoxib + radiation: 0.82 mm$^2$ (0.72/1.01). Afterwards, the tumor dimensions increased in all groups until day 28 control: 9.13 mm$^2$ [6.1/10.45], celecoxib: 5.21 mm$^2$ (3.79/6.2), radiation: 4.35 mm$^2$ (3.26/5.69), celecoxib + radiation: 1.99 mm$^2$ (1.41/4.05)].

Figure 1: Growth of secondary A549 lung carcinomas was analyzed for 28 days by intravital microscopy. The graph depicts the two-dimensional tumor surface (mm$^2$) of the tumors with different treatments on days 7, 14, 21 and 28 after implantation. Median values are represented together with the 25% and the 75% quartiles (n = 6 for each group and time-point). ANOVA, Student-Newman-Keuls-Method, *p<0.05 vs. Control, $\Phi$p<0.05 vs. Radiation, $^+$p<0.05 vs. Celecoxib.

Celecoxib and radiation therapy alone resulted in similar growth behaviors of the tumors. At the end of the experiments the tumor size was significantly reduced in both groups as compared to controls [celecoxib: 5.21 mm$^2$.
(3.79/6.2), radiation: 4.35 mm² (3.26/5.69), control: 9.13 mm² (6.1/10.45), p<0.001 celcoxib vs. control, p<0.001 radiation vs. control]. However, the two-dimensional tumor size significantly increased until day 28 if compared to day 7 (prior to initiation of celcoxib / vehicle treatment) and 14 (prior to single beam irradiation) after implantation. The combination of radiation with celecoxib resulted in a significant reduction of tumor size on day 28 compared to controls, days 7 and 14 after implantation, and compared to the monotherapies [celecoxib + radiation: 0.82 mm² (0.72/1.01), p<0.001 vs. control, p<0.05 vs. celecoxib (day28), p<0.05 vs. radiation (day 28)]. Furthermore, the combination of celecoxib and radiation induced a growth arrest of the tumors after the delivery of irradiation (Fig.1).

Angiogenesis and tumor vascularization

The first newly formed vessels were observed within 6 days after implantation in all tumors followed by a rapid onset of perfusion in these vessels within another 24 hours. Intravital microscopy showed that the angiogenic sprouting originated from the vessels located within the surrounding bone. As shown in figure 2, functional vessel density significantly increased between days 7 and 14 after tumor implantation in all treatment groups. In controls, FVD then remained on the same level during the investigation period of 28 days [day 14: 10.4 mm/mm² (9.8/10.5), day 28: 10.1 mm/mm² (9.8/10.5)]. The therapeutic efficacy of celecoxib and radiation as monotherapies on tumor vascularization was similar. Both therapy regimes resulted in a significant decrease of FVD on day 28 compared to controls on day 28 and compared to day 14 of the same treatment [celecoxib day 14: 9.6 mm/mm² (8.0/10.5), day 28: 7.9 mm/mm² (6.6/8.4), p<0.05; radiation day 14: 10.0 mm/mm² (9.4/11.1), day 28: 8.1 mm/mm² (7.2/8.6), p<0.001]. However, at the end of the experiments, FVD was statistically greater in both groups as compared to the baseline levels on day 7 (p<0.05). Time course experiments showed that the FVD constantly decreased in tumors treated with celecoxib. In irradiated tumors, FVD strongly decreased between days 14 and 21 [10.0 mm/mm² (9.4/11.1) vs. 7.0 mm/mm² (6.5/7.3), p<0.05] and increased again between days 21 and 28 [7.0 mm/mm² (6.5/7.3) vs. 8.1 mm/mm² (7.2/8.6), p<0.05]. The combination of celecoxib and radiation induced a significant decrease of FVD from day 14 to day 28 [day 14: 9.6 mm/mm² (8.0/10.6), day 28: 5.3 mm/mm² (4.9/5.7), p<0.001]. Direct comparison of celecoxib + radiation with the monotherapy regimes showed that
the combination of radiation with celecoxib was superior to both monotherapies in terms of the final functional vessel density on day 28 after tumor implantation. In contrast to the monotherapies the simultaneous treatment with celecoxib and radiation achieved a return of the FVD to the baseline levels measured on day 7.

**Figure 2:** Vascularization of A549 tumor xenografts was analyzed for 28 days by intravital microscopy. (A) The graph depicts the functional vessel densities (FVD) of the tumors with different treatments on days 7, 14, 21 and 28 after implantation. Median values are represented together with the 25% and the 75% quartiles (n = 6 for each group and time-point). ANOVA, Student-Newman-Keuls-Method, *p<0.05 vs. Control, †p<0.05 vs. Radiation, ‡p<0.05 vs. Celecoxib. (B-D) Representative photographs of A549 tumor xenographs from intravital microscopy imaging of the tumor vascularization on day 28; (B) Control, (C) Radiation, (D) Celecoxib + Radiation; scale bars represent 500µm.

**Histology**

Representative H&E stained tissue section of the control group and the experimental groups are shown in figure 3. Typical signs of a malignant tumor growth were observed in controls 28 days after tumor implantation. In
accordance to in vivo findings, the volume of untreated secondary lung carcinomas increased significantly compared to the small pieces (volume 0.5-1.0 mm³) that were initially implanted (Fig. 3A). The implanted tumor piece developed to a large tumor formation that had grown above, below, and into the calvaria. Histology revealed extensive infiltration and resorption of the adjoining bone as signs for typical growth behavior of malignant tumors. However, infiltration into the underlying brain was not observed 28 days after tumor implantation. Compared to controls, the volume of A 549 tumors in all treatment groups was found to be markedly smaller (Fig 3B-D). Consistent to the in vivo findings, the combination of celecoxib and radiation showed markedly smaller tumor volumes at day 28 compared to the monotherapies with celecoxib or radiation alone.

Figure 3: Light micrographs of 4-µm-thick vertical sections through cranial defects, 28 days after tumor implantation. The sections were stained with hematoxylin-eosin. Scale bars represent 500 µm. Tumor borders are marked by arrows. (A) Control (untreated) A549 lung carcinoma. Large tumor formations were found in untreated secondary bone tumors of A549 lung carcinomas. Tumors treated with either Celecoxib (B) or radiation (C) were similar in size and were found markedly smaller as compared to untreated tumors. Tumors treated with radiation and celecoxib (D) exhibited the smallest tumor size at 28 days after tumor implantation.
DISCUSSION

The cox-2 inhibitor celecoxib is known for its antiangiogenic potential, which has been described in various in vitro and in vivo studies and is mainly mediated by the inhibition of the cox-2 enzyme and the downstream expression of the eicosanoid products prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), Thromboxane A\textsubscript{2} (TXA\textsubscript{2}) and Prostacyclin (PGI\textsubscript{2})\textsuperscript{26,42-53}. Celecoxib may also mediate antiangiogenesis by promotion of endothelial cell apoptosis via cox-2 dependent and cox-2 independent mechanisms\textsuperscript{54-58}. Furthermore, substances interfering with the prostaglandin pathway such as cox-2 inhibitors were shown to exert antitumor activities via an inhibition of tumor angiogenesis in vivo\textsuperscript{36,42,43,45,47,59}. The major mode of tumor cell death mediated by radiation is the induction of tumor cell apoptosis. Besides this mechanism non-apoptotic cell death may proceed via necrosis or by genetically programmed mechanisms distinct from classic apoptosis\textsuperscript{60}. Furthermore, irradiation does not only mediate a direct induction of tumor cell death but also seems to promote tumor destruction indirectly by inducing endothelial cell death and by inhibiting angiogenesis\textsuperscript{61,62}.

In the present study, radiation as well as celecoxib showed distinct antivasular properties. Radiotherapy and cox-2 inhibition induced a decrease of the tumor vascularization and an inhibition of tumor growth, without significant differences between the two treatment regimes. The decrease in functional vessel density and the inhibition of tumor growth proceeded simultaneously indicating that the antitumor effects of radiotherapy and celecoxib treatment can be attributed - at least in part - to antiangiogenic mechanisms. However, both monotherapy regimes were not effective enough to stop tumor progression although tumor angiogenesis was successfully suppressed. Interestingly, our time-course data showed that radiation induced a strong decrease of tumor vascularization within the first seven days after single beam radiation. Subsequently however, the functional vessel density partially recovered between days 21 and days 28 of the investigation, which may explain why radiotherapy alone did not effectively stop tumor growth.

Based on findings that the modulation of prostaglandin synthesis can ameliorate the response of malignant primary tumors to radiation\textsuperscript{13,14,19,20} we hypothesized that combining radiation with cox-2 inhibition may increase the radioresponse of secondary bone tumors. Previous studies showed that the continuous administration of different cox-2 inhibitors prior to a single beam radiation of
human tumor xenografts demonstrated to potentiate the tumor response to radiation \textit{in vivo} while the normal tissue was not sensitized to radiation \textsuperscript{19,39,63}. A recent study demonstrated that elevated levels of cox-2 correlate with reduced patient survival after radiation therapy \textsuperscript{64}. This observation indicates that cox-2 and/or its downstream eicosanoid products may protect tumor cells from radiation damage. The radio-protective capacity of cox-2 was further supported by the finding that radiation dose-dependently induced the expression of cox-2 in PC-3 tumor cells \textit{in vitro} \textsuperscript{65}.

The present study demonstrated that, in contrast to the monotherapies with either radiation or celecoxib, the combination of both regimes was capable to halt tumor progression effectively. There was no tumor progression observed following radiation, if the mice bearing the tumors were additionally treated with celecoxib. The profound inhibition of tumor progression was accompanied by a sustained regression of tumor feeding blood vessels. In comparison to the reduction of tumor size and functional vessel density achieved with radiotherapy only, the treatment with radiation and celecoxib reduced tumor vascularization and tumor size by another 57% and 51%, respectively. These results are consistent with the enhanced inhibition of capillary sprouting from rat aortic rings by combined administration of radiation and the cox-2 inhibitor rofecoxib \textsuperscript{61}. Davis \textit{et al.} \textsuperscript{66} suggested that cox-2 derived prostaglandins are important survival factors for malignant tumors and their vasculature after the initial radiation damage. The inhibition of these survival factors with celecoxib enhanced the vascular damage induced by radiation \textit{in vivo} as demonstrated by increased microvessel permeability of the vasculature of Col26 murine colon cancer. In accordance with the studies by Dicker \textit{et al.} \textsuperscript{61} and Davies \textit{et al.} \textsuperscript{66} the present data suggest that celecoxib enhanced the radioresponse of secondary bone tumors by inhibiting angiogenesis within the tumor xenografts.

In conclusion, the simultaneous administration of celecoxib and radiation seems to be a rationale to enhance the therapeutic potential of local radiotherapy of bone metastases. Due to the intrinsic anti-tumor properties of celecoxib this regime offers the advantage to ameliorate the radioresponse of bone metastases locally and accomplish a systemic tumor therapy in non-irradiated regions concomitantly.
ACKNOWLEDGEMENTS

This study was supported by a research grant (F.02.0015) of the MWFK Ba-Wue (Ministry for Science, Research, and Art of the State of Baden-Wuerttemberg, Germany) to AS.

REFERENCES


33. Kucab JE, Lee C, Chen CS, Zhu J, Gilks CB, Cheang M, Huntsman D, Yorida E, Emerman J, Pollak M, and Dunn SE. Celecoxib analogues disrupt Akt signaling,


